Carbon Dioxide Sequestration by *Synechococcus* sp. Strain PCC8806

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ABSTRACT

Expected increases in electricity generation in the near future will require the development of novel carbon sequestration technologies to alleviate associated increases in CO_2 production. Biological sequestration of CO_2 using cyanobacteria represents an attractive technology for this purpose. Some species of cyanobacteria, such as *Synechococcus* sp. strain PCC8806 are able to remove CO_2 from their growth environment by fixation into cellular biomass or via precipitation of $CaCO_3$. The ability of PCC8806 to sequester CO_2 via both mechanisms was tested using 500 ml microcosm studies. Carbon dioxide concentrations of 100 ppmv, 350 ppmv, 1% and an HCO_3^- concentration of 2.5 mM were used for testing. Fixation of CO_2 into biomass by PCC8806 increased with increasing CO_2 concentration and was highest when 2.5 mM HCO_3^- was used as the inorganic carbon source. Growth was minimal in microcosms containing 100 and 350 ppmv CO_2. Precipitation of $CaCO_3$ occurred in the microcosms containing 2.5 mM HCO_3^- and was associated with increasing pH of the growth medium caused by the transport of ions in and out of the cell. These preliminary results indicate that PCC 8806 may be able to remove significant amounts of CO_2 that could be sequestered for long periods of time as sediments on the ocean bottom.

INTRODUCTION

Ice cores from the Antarctic have indicated that atmospheric CO_2 concentrations have been steadily increasing for the past 250 years[1]. The pre-industrial atmospheric CO_2 concentration was near 280 ppm; this concentration has increased to near 365 ppm in 1998. The average increase per year between 1990 and 1999 was 1.5 ppm per year. Anthropogenic carbon dioxide emissions have increased by an average of 1.2% per year during the past 12 years with estimated emissions for 2002 reaching 5.8 billion metric tons of CO_2[2]. Economic growth as well as increased demand due to colder winters and hotter summers indicates that this trend may continue. While the effects of elevated CO_2 levels on global climate are uncertain, there is consensus among scientists that a doubling of atmospheric CO_2 concentrations in the foreseeable future could have a variety of adverse environmental consequences.

The U.S. Department of Energy published a report in 1999 titled, "Carbon Sequestration Research and Development,"[3] which details research needs related to the capture and sequestration of CO_2. Part of this reports focuses on developing a roadmap for carbon capture and sequestration research and development, which includes the development of enhanced ocean processes and advanced biological adsorption technologies. Carbon capture through the activity of cyanobacteria in the genera *Synechocystis* and *Synechococcus* represents a potentially powerful method to sequester CO_2 in biomass and in the form of $CaCO_3$. Understanding carbon transport and fixation in these microorganisms will allow for better control of CO_2 removal by these organisms resulting in the potential for developing practical technologies from this fundamental knowledge.

BACKGROUND

In considering the earth's carbon distribution, it is relatively easy to ascertain that much of the carbon that is represented in the global carbon cycle is sequestered (for the most part permanently) primarily as calcium and calcium-magnesium carbonates.[4] In many cases, the carbonates are of biogenic origin, some precipitated by bacteria, cyanobacteria, and fungi. Calcium or calcium-magnesium carbonates are precipitated by numerous mechanisms, one of which is photo- and chemosynthetic autotrophy in the presence of Ca and Mg counterions. Precipitation of carbonaceous sediments by oxygenic photosynthetic bacteria classified as cyanobacteria is of particular interest because cyanobacteria represent a diverse group of photosynthetic prokaryotes that exhibit versatile physiology and wide ecological tolerance that has allowed them competitive success in a broad spectrum of environments. They are found in numerous terrestrial environments; but, more importantly, they are common in freshwater bodies and the cyanobacterium, *Synechococcus*, contributes up to 50% of the chlorophyll a biomass in oligotrophic oceans.[5] In addition, marine cyanobacteria are responsible for an estimated 20 – 40% of carbon fixation in oceans.[6]

The purpose of this study was to determine the effect of CO_2 concentration on cell growth and changes of parameters important to $CaCO_3$ formation and nucleation on the cell surface. In addition, initial tasks to monitor expression of genes related to bicarbonate transport using reverse transcriptase real-time PCR are discussed. These transporters are important because they catalyze changes in cell surface pH necessary for $CaCO_3$ formation when transporting carbon for fixation into cellular materials.

MATERIALS AND METHODS

Synechococcus sp. PCC strain 8806 was grown at various CO_2 concentrations to determine the effect of these differences on growth and other factors that effect $CaCO_3$ precipitation. Carbon dioxide concentrations tested were 100 and 350 ppmv, 1%; in addition, 2.5 mM bicarbonate was studied. A starting concentration of 1 X 10^6 cells/ml, which is equivalent to an OD_{600} of 0.095 was used. To eliminate residual CO_2, the medium and bottle headspace were purged with nitrogen, then oxygen was reintroduced to a final concentration of 21% (v/v). Cyanobacterial cells were inoculated into 200 ml of Modified 617 growth medium containing no carbonate and amended with 3.4mM calcium. Modified 617 medium consisted of the following: 17.65mM $NaNO_3$, 0.30mM $MgSO_4 \cdot 7H_2O$, 3.4mM $CaCl_2 \cdot 2H_2O$, 171mM NaCl, 0.18mM K_2HPO_4, 0.03mM citric acid, 0.03mM ferric ammonium citrate, 0.003mM disodium magnesium EDTA, 1 ml Trace metal mix A5+Co (Trace metal mix A5+Co ($g \cdot l^{-1}$): H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.222, $Na_2MoO_4 \cdot 2H_2O$ 0.390, $CuSO_4 \cdot 5H_2O$ 0.079, $Co(NO_3)_2 \cdot 6H_2O$ 0.049), and 20 µg Vitamin B_{12}. The 500 ml sealed serum vials were then respiked with the various concentrations of CO_2 and $NaHCO_3$. The vials were incubated at 25°C, shaken at 120 rpm, with a 12 hour light/dark cycle, and photon irradiation of 28 µmol $m^{-2}s^{-1}$.

CO_2 in the headspace of the microcosms was measured using gas chromatography. The gas chromatograph was a Hewlett-Packard 5890 equipped with a thermal conductivity detector and a Chrompack Poraplot Q column (0.32 µm dia, 27.5 m length). The carrier gas was helium at a flowrate of 2 ml min^{-1}, with a split ratio of 1:10. The injection temperature was set at 125 °C, the detector temperature was set at 250 °C, and the oven temperature was maintained at 30 °C.

The optical density (O.D.) of the cultures was measured on a Shimadzu UV160U spectrophotometer at a wavelength of 600 nm at various time points through the course of the experiment. A 2ml aliquot was removed from the sample bottles and placed in a cuvet. If the O.D. was greater than 1.0, the sample was diluted 1:10 and was re-measured on the spectrophotometer.

The pH was measured on a 0.5ml aliquot that was removed from the sample bottles and filtered through a 0.2µm nylon acrodisc syringe filter at various time points during the course of the experiment. The bottles were re-spiked with CO_2 or $NaHCO_3$ to the original concentration of each bottle when the measured levels were zero, which was daily or every other day.

At the conclusion of the experiment the cells from the CO_2 microcosms were harvested, biomass normalized, and total RNA was extracted using a Qiagen RNeasy RNA extraction kit. Instructions in the kit were followed during extraction. After extraction, the RNA was treated with DNase to remove any DNA contamination present in the samples.

RESULTS AND DISCUSSION

Microcosm experiments were run to determine the effect of CO_2 concentration on growth and factors that would affect precipitation of $CaCO_3$ by the cyanobacteria *Synechococcus* sp. strain PCC 8806. Three concentrations of CO_2 (100 and 350 ppmv and 1% v/v) and 2.5 mM HCO_3^- were tested. Parameters that were monitored were CO_2 removal, oxygen production, pH and growth via measurements of optical density at a wavelength of 600 nm. The experiments were run for approximately 23 days and at the end of the experiment, RNA was extracted to determine the effect of CO_2 concentration on expression of genes related to inorganic carbon transport.

Carbon dioxide and oxygen concentrations from the microcosm used to test 100 ppmv CO_2 can be seen in Figure 1A. The CO_2 concentration started out higher than the target concentration of 100 ppmv and was probably caused by residual CO_2 added when the microcosm was inoculated. After two days of incubation, CO_2 levels in the headspace remained below the limits of detection by the GC method being used. Concentrations of oxygen in the headspace increased slightly at the beginning of the experiment and then stabilized near 21%.

Figure 1B shows the effect of inorganic carbon concentrations on growth and pH. The initial pH in the microcosm was approximately 8.5, but pH was not measured again until day 5 of the experiment. The pH increased to nearly 9.5 and then stabilized near 8.9. The initial increase in pH correlates to a sharp increase in optical density, indicating that the culture was growing. After day 6, the optical density of the culture decreased to an average density of 0.12 which is equivalent to approximately 1.7×10^6 cells/ml. The apparent cause of the sharp decrease in cell density is not known. When comparing pH to optical density it is apparent that the drop in pH correlates very closely to the drop in cell number.

As with the microcosm used to test 100 ppmv CO_2 the initial CO_2 concentration in the microcosms used for testing 350 ppmv CO_2 was high. This was again attributed to carryover during inoculation (Figure 2A). Once again, after 2 days of growth, there was no CO_2 noted in the headspace for most of the experiment even though the vials were respiked on a regular basis. Oxygen levels in the headspace increased to near 25% and remained at this concentration for the duration of the experiment.

Cell density increased very gradually over the duration of the experiment following a sharp increase during the first 7 days of the experiment (See Figure 2B). This initial jump in cell density may have been due to the higher concentration of CO_2 at the beginning of the experiment. The rate of growth following day 2 better represents growth when the CO_2 concentration is 350 ppmv. The maximum cell density achieved was near 1.8×10^6 cells/ml. Changes in pH followed a similar pattern, a sharp increase during the first 7 days of the experiment, followed by more gradual increases at the end. A maximum pH of 9.3 was achieved during the experiment.

More significant changes in the oxygen concentration were noted when the CO_2 concentration was elevated to 1% v/v, as demonstrated in Figure 3A. During the test, the oxygen concentration increased from 17% at the beginning of the experiment to nearly 37% at the end. Unlike the lower CO_2 concentrations tested, CO_2 was still present in the headspace on day two of sampling. By day six, the CO_2 in the headspace had been depleted and only increased slightly on day 13 as additional CO_2 was added to the microcosm.

Removal of large amounts of CO_2 led to substantial increases in pH and cell density (Figure 3B). The pH in the microcosm increased from the initial value of 8.5 to 10.44 by day 20 of the experiment. The optical density in the culture increased from 0.046 upon inoculation to 0.751 at the conclusion of the experiment. These results indicate that significant changes in pH are associated with increased uptake of CO_2 and associated growth of the cell.

The most drastic increase in oxygen levels was demonstrated in the microcosms that received 2.5 mM HCO_3^-, as seen in Figure 4A. Oxygen levels in the headspace increased from approximately 16% to above 47 % by the end of the experiment, indicating that there was rapid photosynthesis occurring in the culture. Since the inorganic carbon source was added as HCO_3^-, there was no CO_2 measured initially because the microcosm had not come to equilibrium related to inorganic carbon species. On day 2 there was still nearly 400 ppmv CO_2 present in the headspace but levels decreased to near zero by day six of the experiment.

Changes in pH were similar when the cells were grown with 2.5 mM HCO_3^- (Figure 4B) and 1% CO_2; however, cell growth in the 2.5 mM HCO_3^- microcosm was substantially higher than growth seen when 1% CO_2 was tested. The pH increased from the initial value of 8.5 to a maximum value of 10.7 on day 13 of the experiment. The optical density of the culture increased to approximately 1.2 from the initial value of roughly 0.05. An optical density of 1.2 represents a cell density of approximately 1.5×10^7 cells/ml.

Visual examination of the microcosms containing 1% CO_2 and 2.5 mM HCO_3^- at the conclusion of the experiment indicated the presence of a white precipitate assumed to be $CaCO_3$ in the liquid. The presence of this precipitate in the microcosms containing 100 ppmv and 350 ppmv CO_2 was not noted. Precipitation of CO_2 in the microcosms receiving 1% CO_2 and 2.5 mM HCO_3^- was probably initiated due to the increase in pH above the second equilibrium constant for inorganic carbon, which would have led to the presence of CO_3^{2-} for inclusion into the growing $CaCO_3$ crystal. Changes in calcium concentrations were not measured during the experiment.

At the conclusion of the experiment, biomass was harvested by centrifugation and the concentration of cells taken from the CO_2 microcosms was normalized and the RNA was extracted using the RNeasy RNA Extraction kit from Qiagen. Extraction of RNA from the culture from the microcosm used to test 2.5 mM HCO_3^- was problematic so a sufficient RNA sample could not be obtained. RNA concentrations extracted from the 100 ppmv, 350 ppmv and 1% CO_2 microcosms were 39.2 µg RNA/ml, 16.8 µg RNA/ml and 38 µg RNA/ml, respectively. These numbers do not represent a quantitative measure of total RNA present in the cell due to potential differences in extraction efficiencies between the samples.

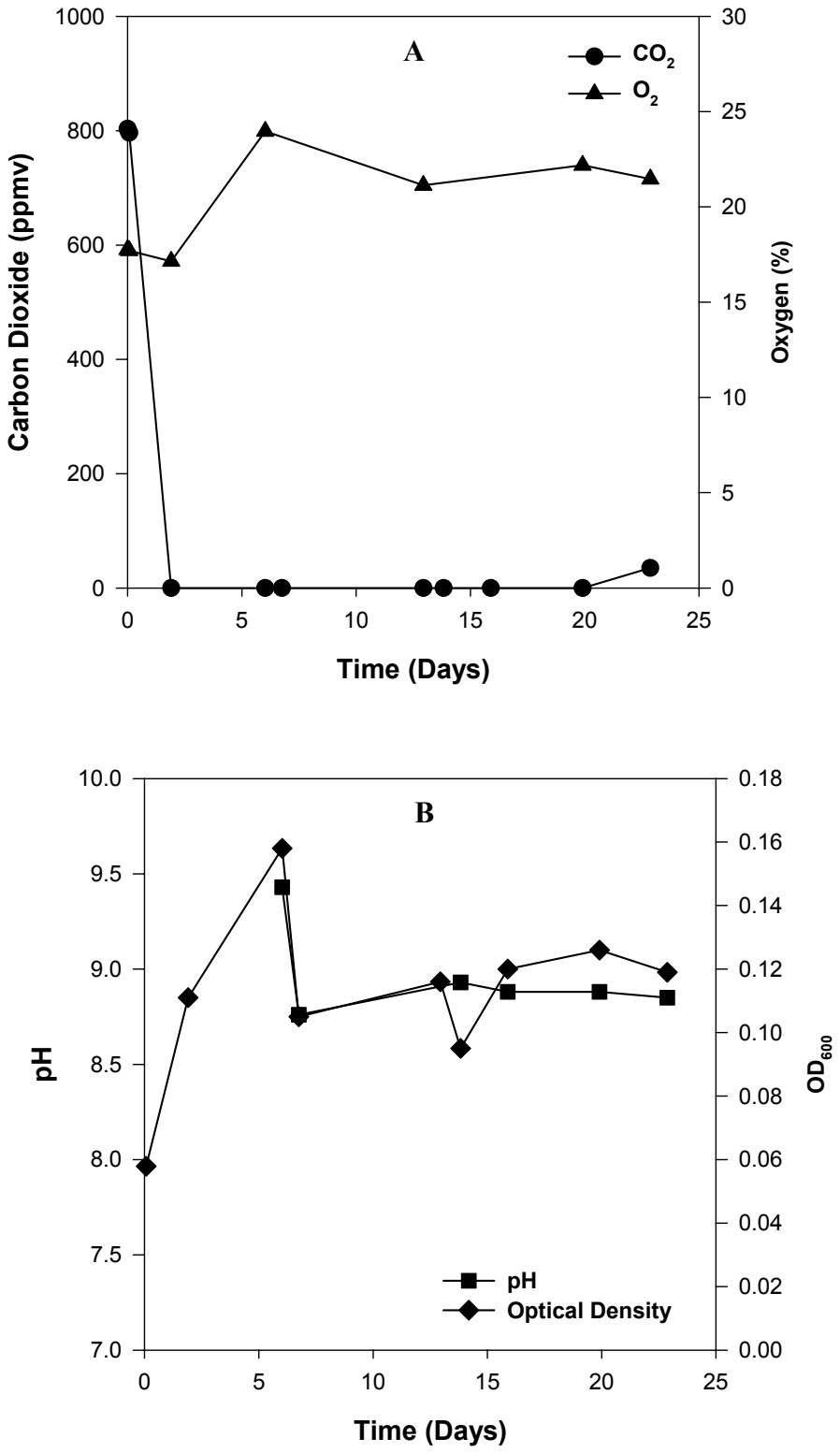

Figure 1. Experimental results from microcosm experiments to determine the effect of 100 ppmv CO_2 on growth and changes in other culture parameters that would affect the sequestration of CO_2. (A) Figure showing CO_2 removal and oxygen production. (B) Figure showing cell growth as a function of optical density measured at 600 nm and changes in pH.

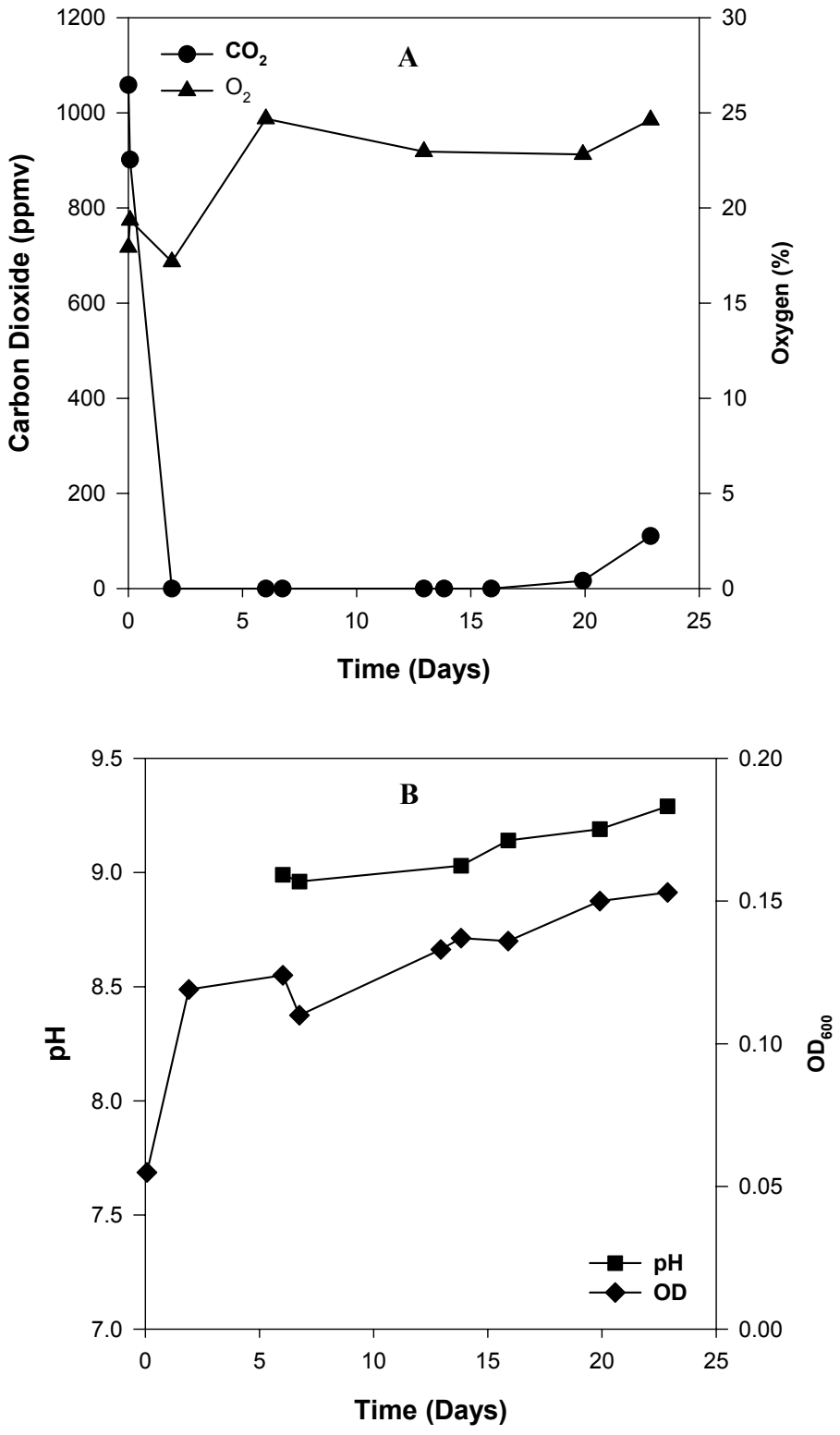

Figure 2. Experimental results from microcosm experiments to determine the effect of 350 ppmv CO_2 on growth and changes in other culture parameters that would affect the sequestration of CO_2. (A) Figure showing CO_2 removal and oxygen production. (B) Figure showing cell growth as a function of optical density measured at 600 nm and changes in pH.

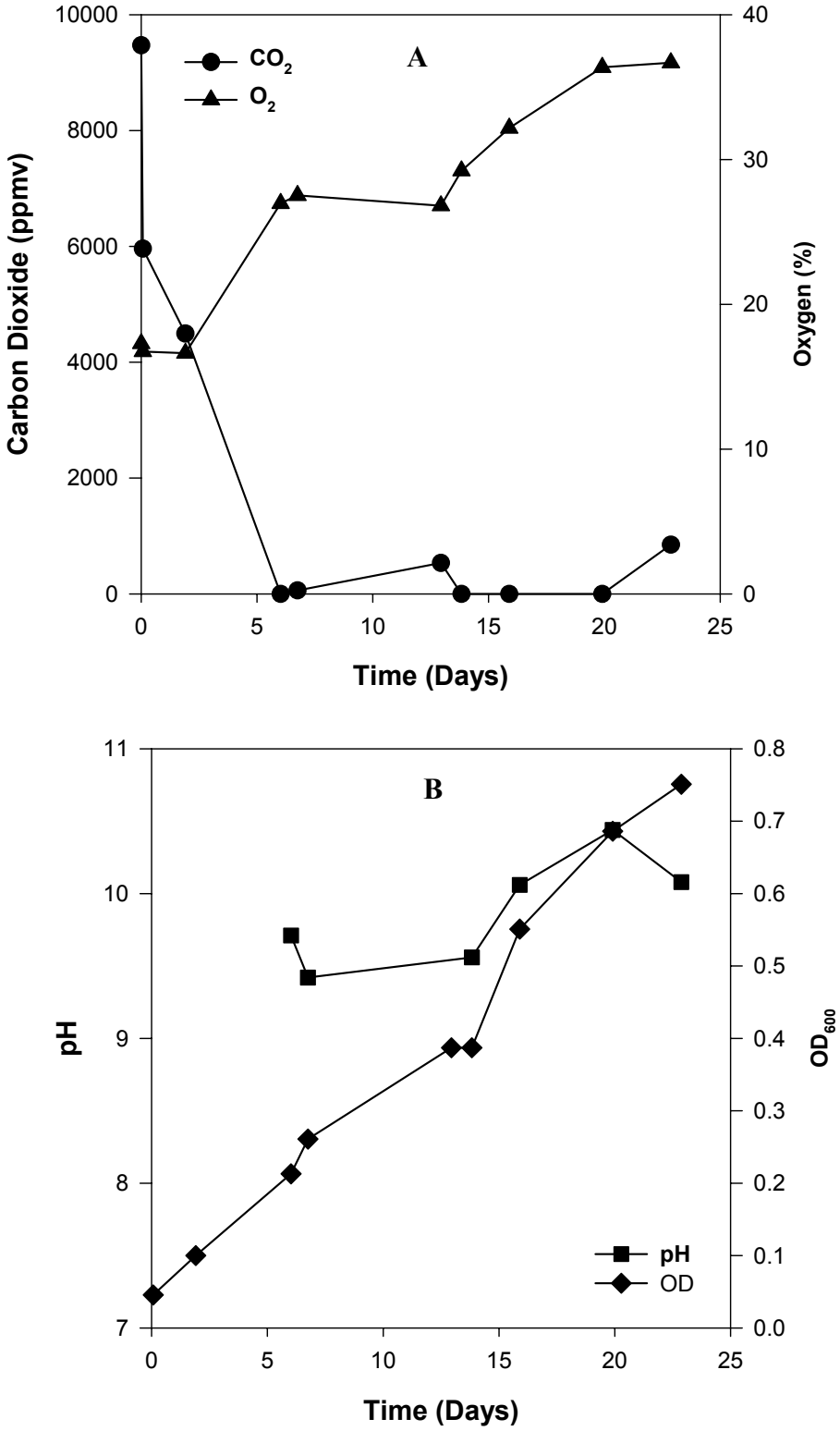

Figure 3. Experimental results from microcosm experiments to determine the effect of 1% CO_2 on growth and changes in other culture parameters that would affect the sequestration of CO_2. (A) Figure showing CO_2 removal and oxygen production. (B) Figure showing cell growth as a function of optical density measured at 600 nm and changes in pH.

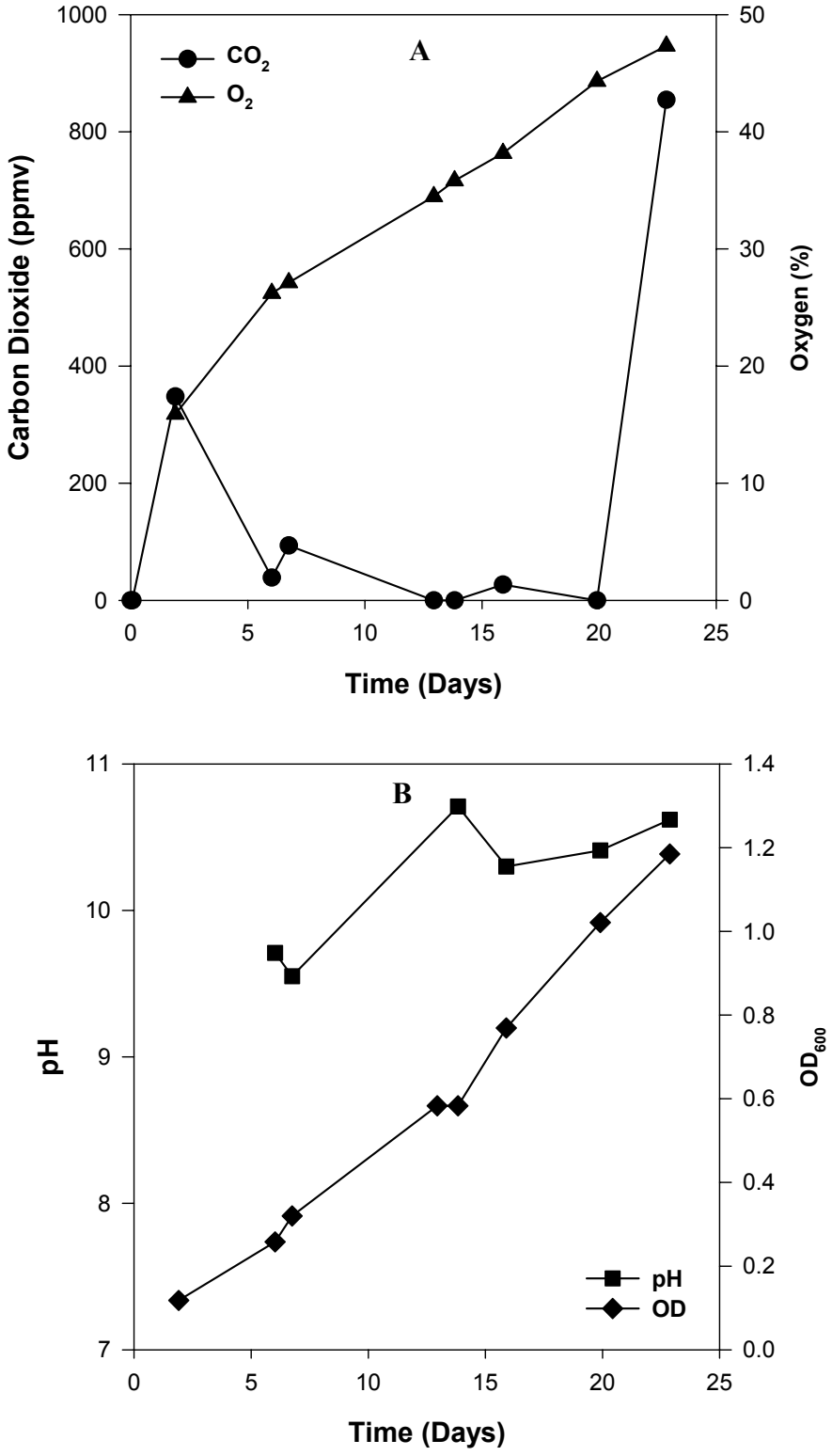

Figure 4. Experimental results from microcosm experiments to determine the effect of 2.5 mM HCO_3^- on growth and changes in other culture parameters that would affect the sequestration of CO_2. (A) Figure showing CO_2 removal and oxygen production. (B) Figure showing cell growth as a function of optical density measured at 600 nm and changes in pH.

Oligonucleotide primes were developed for use in monitoring changes in expression of genes involved in inorganic carbon transport. Genes that were targeted for this research were *cmpA* and *cmpB*. *CmpA* encodes a 42 kD protein involved in binding of bicarbonate for transport into the cell.[7] Research has shown that when cyanobacteria are transferred from conditions of high CO_2 to low CO_2 that expression of this gene is induced.[8,9] *CmpB* is gene within the same operon as *cmpA* and encodes a membrane bound protein in the same inorganic carbon transport protein complex. It has not been determined whether expression of this gene is induced under similar conditions.

Primers were developed from known sequences for these genes that were obtained by using the Basic Logical Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI). These sequences were entered into the PrimerQuest online primer design program from Integrated DNA Technologies. Primers suggested by the program were run through NetPrimer to rule out the formation of primer dimers and hairpins. Sequences and chemical properties for each primer can be seen in Table 1.

Table 1. Oligonucleotide primers synthesized to monitor expression of inorganic carbon transport genes.

Name	Sequence (5' – 3')	T_m (°C)	GC Content (%)
Synechococcus cmpA 3 F	GTTATCAGCCTTATCGGTCTGG	56.1	50
Synechococcus cmpA 3 R	GGAGATGCCTCTAAAGATGGAC	55.9	50
Synechococcus cmpB 2 F	GTACCTAATGAAGGTGGTCTTGCC	58.8	50
Synechococcus cmpB 2 R	CCAGATAAAGAAGCCAATGCCGAC	59.8	50

Primers will be optimized for use in reverse transcriptase, real-time PCR assays. Primer optimization is being performed by probing DNA extracted from *Synechococcus* sp. strain PCC 8806 grown under atmospheric conditions (i.e., ~365 ppmv CO_2).

CONCLUSIONS

Results from the research demonstrate that *Synechococcus* sp. strain PCC 8806 could be used to remove substantial amounts of CO_2 during growth. CO_2 utilized could be removed by two mechanisms; first by fixation into biomass that was evident by growth of the culture at all of the CO_2 concentrations tested. Second, inorganic carbon transport mechanisms as well as ion transport by PCC 8806 leads to increased pH which is the initial step required for nucleation and eventual precipitation of $CaCO_3$. Formation of $CaCO_3$ crusts on the cell surface as demonstrated at low inorganic carbon concentrations would lead to precipitation of $CaCO_3$ and the encased cell that results in sequestration of atmospheric CO_2 into $CaCO_3$ muds on the bottom of the ocean. As the $CaCO_3$ muds are buried, the carbon would be locked up for long periods of geological time.

Future research will be performed to optimize the primers to monitor expression of genes related to carbon transport by *Synechococcus* sp. strain PCC 8806. Microarray experiments will also be run using Intelligene CyanoCHIP Microarrays to determine other genes expressed during inorganic carbon transport at various inorganic carbon concentrations. In addition, microcosms will be set up to more closely monitor portioning of inorganic carbon between biomass and $CaCO_3$.

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